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Direct evidence that pyrophosphate:fructose-6-phosphate phosphotransferase can act as a glycolytic enzyme in plants

Wolf-Dieter Hatzfeld, Jane Dancer and Mark Stitt

Lehrstuhl für Pflanzenphysiologie, 8580 Bayreuth, FRG

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Experiments were carried out to provide direct evidence that pyrophosphate:fructose-6-phosphate phosphotransferase (PFP) can operate as a glycolytic enzyme in some circumstances. A large increase in the rate of glycolysis was produced by adding uncoupler and alkalinising the medium of heterotrophic *Chenopodium rubrum* cells. Initially, a marked decrease in phospho*enol*pyruvate, 3-phosphoglycerate and hexose phosphates occurred, but no change in fructose 2,6-bisphosphate or inorganic pyrophosphate was observed. However, a gradual increase in the rate of O_2 uptake during the subsequent 5 min was accompanied by an increase in fructose 2,6-bisphosphate and a decrease in pyrophosphate, providing evidence that activation of PFP contributes to the increase in rate of glycolysis. This was accompanied by partial recovery of the adenine nucleotide energy status.

Pyrophosphate:fructose-6-phosphate phosphotransferase; Pyrophosphate; Fructose-2,6-biphosphate; Glycolysis; (Plant)

1. INTRODUCTION

In most eukaryotes, Fru2,6P_2 activates the ATP-dependent PFK and acts as a 'glycolytic signal' [1,2]. However, Fru2,6P_2 has no effect on PFK from higher plants but, instead, activates an enzyme called PFP [3–5]. PFP catalyses the reversible phosphorylation of Fru6P using PP_i as phosphoryl donor. In analogy to other eukaryotes, it has been suggested that PFP is a glycolytic enzyme and that a rise in Fru2,6P_2 stimulates glycolysis in plants by activation of this enzyme [6–15]. However, there is still no direct evidence that PFP can act as a glycolytic enzyme. Firstly, the reaction catalysed by PFP is near to equilibrium in vivo [16,17] and Fru2,6P_2 acts to stimulate both forward and reverse reactions in a similar manner [18]. This means that PFP could catalyse the reverse reaction. Indeed, many workers have suggested this to be its function, either during gluconeogenesis [19] or to generate PP_i [20–22]. Secondly, there are no experimental data which satisfy the following criteria for the role of PFP in plant glycolysis in vivo: (i) Fru2,6P_2 must increase and this rise should precede the increase in flux; and (ii) the increase in Fru2,6P_2 should be accompanied by a decline in PP_i. A drop in Fru6P alone [12] is not an adequate criterion, since this would also be observed after activation of PFK.

The following experiments were carried out on heterotrophic cell suspensions of *Chenopodium rubrum* in order to allow easy monitoring of rapid changes in fluxes and metabolites. We added low concentrations of uncoupler and simultaneously alkalinised the medium to achieve marked stimulation of respiration and dark fixation. The results show that activation of PFP by rising Fru2,6P_2 can indeed lead to the activation of glycolysis in plant cells.

Correspondence address: M. Stitt, Lehrstuhl für Pflanzenphysiologie, 8580 Bayreuth, FRG

Abbreviations: ACES, 2-[(2-amino-2-oxoethyl)amino]ethanesulfonic acid; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; Fru1,6/2,6P_2, fructose 1,6-/2,6-bisphosphate; Fru/Glc6P, fructose/glucose 6-phosphate; PEP, phospho*enol*pyruvate; PFP, pyrophosphate:fructose-6-phosphate phosphotransferase; PGA, 3-phosphoglycerate; P_i, inorganic phosphate; PP_i, inorganic pyrophosphate

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2. MATERIALS AND METHODS

Heterotrophic *C. rubrum* cell suspension cultures were obtained from a photoautotrophic culture [23] by growing cells in darkness, and supplementing them with glucose. Every 13–14 days, 0.5 g fresh wt of cells were transferred to 80 ml fresh Murashige-Skoog medium containing 100 mM glucose. Cells were taken 9 days after transfer, when they had exhausted the exogenous glucose but still contained substantial stored carbohydrate.

Oxygen uptake was measured at 20°C in an O_2 electrode (Hansatech, Kings Lynn, England) using 1 ml (50 mg fresh wt of cells) of the suspension (medium pH 5.5 ± 0.3). Following a short period to allow equilibration, 15 m ACES (pH 7.3) and 0.5 μM CCCP were added. The rate of $H^{14}CO_3$ incorporation (dark fixation) was measured by incubation of 1 ml cells for 30 s with 2 mM $H^{14}CO_3$ (6.7×10^9 Bq·mol^{-1}) in a scintillation beaker, addition of 1 ml of 2 N HCl and evaporation of the mixture to dryness. The commercially available $NaH^{14}CO_3$ was purified to remove acid-stable contaminants [24].

Samples for metabolite analysis were prepared in parallel with measurements of respiration using cells at an identical density and under the same conditions. An aliquot of 300 mg fresh wt was taken, most of the medium removed through a paper filter using a water suction pump, and the filter and adhering layer (1–2 mm thick) of cells then frozen in liquid N_2. Extracts were prepared and assayed for Fru2,6P_2 and phosphate esters as in [25]. Adenine nucleotides were assayed as in [26]. PP_i was extracted in trichloroacetic acid and determined according to [17]. All metabolite measurements were carried out using a Sigma ZFP 22 dual-wavelength photometer.

Reliability of the extraction and analysis was checked by including small amounts of metabolites (equivalent to the endogenous levels in the cells) in the deproteinising cocktail added to cell samples. Parallel samples received no additional biochemicals. Comparison of the metabolites found in both sets of extracts gave the following values for the recovery of added biochemicals via extraction with $CHCl_3/CH_3OH$ (expressed as percentage of the amount added): Fru2,6P_2, 84; Glc6P, 93; Fru6P, 112; Glc1P, 85; UDPGlc, 85; 3 PGA, 82; PEP, 72; Fru1,6P_2, 79; triose phosphates, 80; pyruvate, 70; ATP, 92; ADP, 91; AMP, 73; PP_i, 73. All represent the means of at least 3 replicates, the SE being less than 11% of the mean.

Extracts for measuring enzyme activities were prepared by homogenization of 150 mg fresh wt cells in 2 ml extraction medium containing 100 mM Hepes (pH 7.6), 5 mM $MgCl_2$, 1 mM EDTA, 5 mM mercaptoethanol, 0.1% (w/w) bovine serum albumin, and 50 μg·ml^{-1} phenylmethylsulfonyl fluoride at 4°C, followed by centrifugation for 5 min at 2000 × g. PFP was assayed in the forward (PP_i-consuming) direction in 75 mM Hepes (pH 7.5), 2 mM Mg acetate, 7.5 mM Fru6P, 10 μM Fru2,6P_2, 0.15 mM NADH, 1 U·ml^{-1} triosephosphate isomerase, 1 U·ml^{-1} glycerol-3-P dehydrogenase, the reaction being initiated by addition of 0.25 mM PP_i. PFK was assayed using 50 mM Hepes (pH 7.9), 15 mM $MgCl_2$, 0.1 mM NADH, 5 mM Fru6P, 1 U·ml^{-1} aldolase, 1 U·ml^{-1} triosephosphate isomerase, 1 U·ml^{-1} glycerol-3-P dehydrogenase, and the reaction started by adding 0.25 mM ATP. Fru1,6Pase was assayed in 50 mM Mops buffer (pH 6.9), 5 mM $MgCl_2$, 0.2 mM NADP, 3.5 U·ml^{-1} phosphoglucose isomerase and 2 U·ml^{-1} glucose-6-phosphate dehydrogenase, initiation of the reaction being via the addition of 50 μM Fru1,6P_2. This Fru1,6P_2 concentration is 10-fold above the K_m of the cytosolic enzyme. PFP shows negligible activity under these conditions, which give a maximal estimate of Fru1,6Pase activity.

3. RESULTS AND DISCUSSION

When the medium pH was increased from pH 5.5 to 7.3, an increase in proton extrusion was observed and dark fixation rose 4-fold without altering the rate of respiration (not shown). When uncoupler was added to cells, rapid doubling (within 2 min) of the rate of respiration occurred (not shown). By adding uncoupler and simultaneously alkalinising the medium, we stimulated O_2 uptake and dark fixation, thereby achieving a large increase in the rate of glycolysis (fig.1A). Oxygen uptake and dark fixation are expressed as nmol hexose consumed·g^{-1} fresh wt·min^{-1}, in order to show the demand each places on glycolysis (one hexose molecule is required for every 6 molecules of O_2 respired, or for every 2 molecules of HCO_3 fixed via PEPCX). These additions therefore led to 3-fold stimulation of glycolysis within 10 min.

The process of the response can be divided into two stages. During the first minute, the rate of glycolysis almost doubles, due mainly to the increased rate of dark fixation (fig.1B). No change occurs in Fru2,6P_2 or PP_i (fig.1B). However, hexose phosphates decrease (fig.1C), PGA and PEP fall dramatically (fig.1D) and the adenine nucleotide status collapses, as revealed by the decreased ATP/ADP ratio (fig.1F). Evidently, strong activation of PEP utilisation takes place. The resulting collapse in PEP concentration subsequently leads to activation of PFK, since the enzyme from plants is very sensitive to inhibition by this metabolite [27].

Between 1 and 8 min, the rate of respiration continued to rise, reaching about double the initial value. This increase was accompanied by a rise in Fru2,6P_2, suggesting that activation of PFP is involved in this gradual increase in the rate of respiration. Assuming the cytosol constitutes about 10% of the cell volume, Fru2,6P_2 will increase from about 0.5 to 6 μM. Under optimal conditions, PFP is already activated by nanomolar Fru2,6P_2 concentrations [4,5]. However, the affinity for Fru2,6P_2 will be much lower in vivo in the

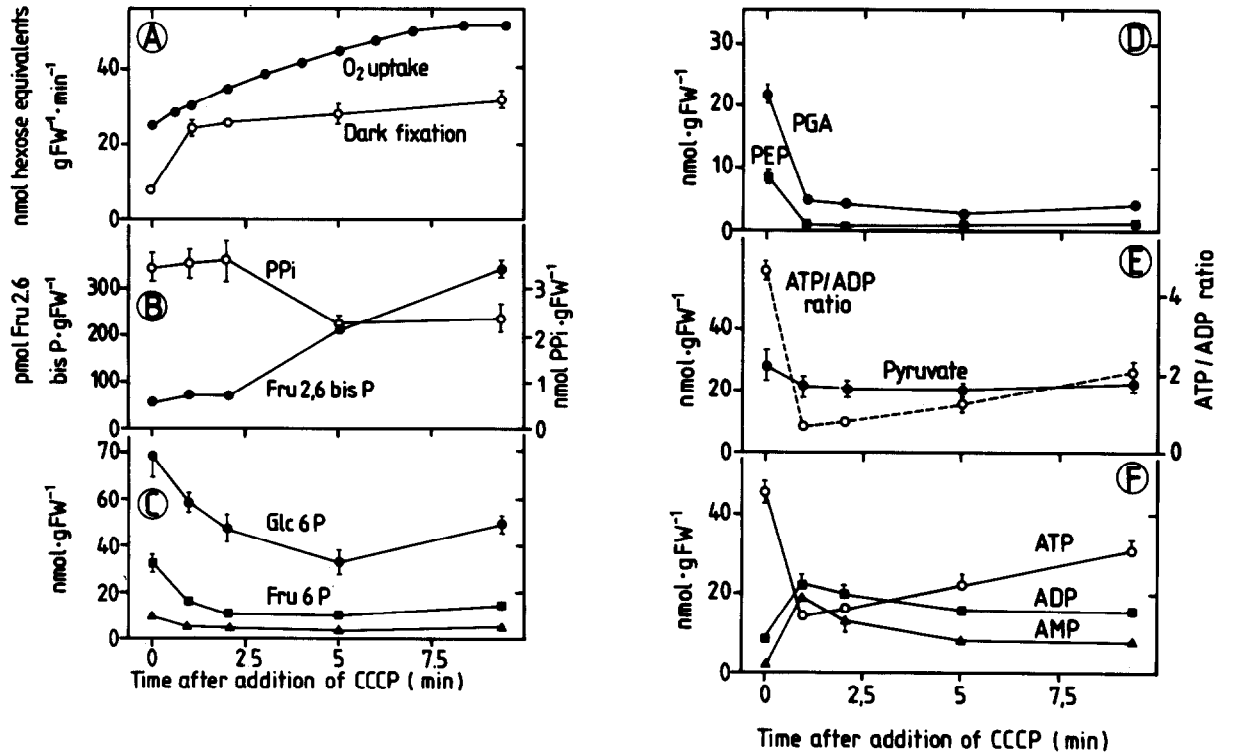

Fig.1. Changes in respiration, dark fixation and metabolites. At time zero, 0.5 μM CCCP was added, and the pH of the medium was increased from pH 5.5 to 7.3; (A) O_2 uptake (●) and dark fixation (○); (B) Fru2,6P_2 (●) and PP_i (○); (C) Glc6(●), Fru6P (■) and Glc1P (▲); (D) PGA (●) and PEP (■); (E) pyruvate (●) and ATP/ADP ratio (○); (F) ATP (○), ADP (■) and AMP (▲).

presence of P_i, anions and various phosphorylated intermediates [28,29].

Activation of PFP could stimulate glycolysis by (i) providing additional glycolytic capacity or (ii) generating PP_i, which can then be used to mobilise sucrose via sucrose synthase and UDP-glucose pyrophorphorylase. Two lines of evidence show that PFP acts as a glycolytic enzyme in our experiment. Firstly, the increase in Fru2,6P_2 is accompanied by a decline in PP_i (fig.1B), as would be expected for increased PP_2 consumption. If PFP were acting to produce PP_i, we would expect PP_i to recover as Fru2,6P_2 increases. Secondly, these cells contained large pools of free glucose (11 ± 4 μmol·g^{-1} fresh wt), fructose (10 ± 3 μmol·g^{-1} fresh wt) and starch (7 ± 2 μmol hexose·g^{-1} fresh wt) but relatively little sucrose (2 ± 1 μmol·g^{-1} fresh wt). It may also be noted that the cells contained negligible fructose-1,6-bis-phosphatase (0.033 μmol·g^{-1} fresh wt·min^{-1}) compared to PFP or PFK (1.2 and 0.4 μmol·g^{-1} fresh wt·min^{-1}, respectively). They also contained negligible Fru1,6P_2. It is therefore unlikely that Fru2,6P_2 acts to stimulate glycolysis by inhibiting Fru1,6Pase.

The increase in Fru2,6P_2 is a consequence of the decrease in PGA (fig.1D), which is a powerful inhibitor of the plant Fru6P,2-kinase [30,31]. The general decline of phosphorylated metabolites (fig.1C–E) also signifies that P_i will increase. This will also contribute to the increase in Fru2,6P_2, since P_i activates Fru6P,2-kinase, acting antagonistically to PGA, and inhibits Fru2,6P_2ase [30,31]. The response of Fru2,6P_2 is delayed as its concentration depends on the balance between the rates of synthesis and degradation and a finite time must elapse before a new steady concentration is reached.

During this slow rise in respiration the hexose phosphates (fig.1C), and PGA and PEP (fig.1D) remained low, and Fru1,6P_2 and triose-P remained below the level of detection (1 nmol·g^{-1} fresh wt).

However, there was a gradual recovery in adenine nucleotide energy status, as demonstrated by the rising ATP/ADP ratio (fig.1E) and the falling level of AMP (fig.1F). This suggests that the additional glycolytic capacity provided by PFP supports a higher rate of respiration which, in turn, allows the partial recovery of adenine nucleotide status under these extreme conditions. This bears some resemblance to a suggestion made by Mertens et al. [15] who observed a correlation between $Fru2,6P_2$ and the ATP/ADP ratio during studies of anaerobiosis. However, they did not provide any direct evidence that the rate of glycolysis had in fact increased, indeed it may have decreased [32], nor did they present results directly demonstrating that PFP had been activated in their experiments.

Our results also reveal that the energy status of the adenylates and PP_i can alter independently. This underlines the potential significance of PP_i as an energy system in the cytosol of plants, and raises the question of the source of PP_i. Although PP_i could be generated by PFP when it operates in the reverse direction, an alternative source of PP_i is needed when PFP acts as a glycolytic enzyme. Further studies will be needed to identify the source of PP_i under these conditions, one possibility being the reversal of the tonoplast PP_i-dependent proton pump.

In conclusion, our results show that PFP can operate as a glycolytic enzyme in plants under conditions where a large and rapid increase in the rate of glycolysis is required, and also that PP_i can act as an energy donor in plants.

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REFERENCES

[1] Van Schaftingen, E. (1987) Adv. Enzymol. Related Areas Mol. Biol. 59, 315–395.
[2] Hue, L. and Rider, M. (1987) Biochem. J. 245, 313–324.
[3] Sabularse, C.D. and Anderson, R.L. (1981) Biochem. Biophys. Res. Commun. 100, 1423–1429.
[4] Cseke, C., Weenden, N.F., Buchanan, B.B. and Uyeda, K. (1982) Proc. Natl. Acad. Sci. USA 79, 4322–4326.
[5] Van Schaftingen, E., Lederer, B., Bartrons, R. and Hers, H.-G. (1982) Eur. J. Biochem. 129, 191–195.
[6] Van Schaftingen, E. and Hers, H.-G. (1983) FEBS Lett. 164, 195–200.
[7] Wu, M.-X., Smyth, D.A. and Black, C.C. (1984) Proc. Natl. Acad. Sci. USA 81, 5051–5055.
[8] Kruger, N.J. and Beevers, H. (1985) Plant Physiol. 77, 358–364.
[9] Huber, S.C. (1986) Annu. Rev. Plant Physiol. 37, 233–246.
[10] Cseke, C.C. and Buchanan, B.B. (1986) Biochim. Biophys. Acta 853, 43–63.
[11] Stitt, M., Cseke, C. and Buchanan, B.B. (1986) Plant Physiol. 80, 246–249.
[12] Hedrich, R., Stitt, M. and Raschke, K. (1985) Plant Physiol. 79, 977–982.
[13] Baysdorfer, C., Sicher, R.C. and Kremer, D.F. (1987) Plant Physiol. 84, 766–770.
[14] Mertens, E., Marcellin, P., Van Schaftingen, E. and Hers, H.-G. (1987) Eur. J. Biochem. 167, 579–583.
[15] Mertens, E., Van Schaftingen, E. and Hers, H.-G. (1987) FEBS Lett. 221, 124–128.
[16] Edwards, J. and Ap Rees, T. (1986) Phytochemistry 25, 2033–2039.
[17] Weiner, H., Stitt, M. and Heldt, H.W. (1987) Biochim. Biophys. Acta 893, 13–21.
[18] Stitt, M. and Vasella, A. (1988) FEBS Lett. 228, 60–64.
[19] Fahrendorf, T., Holtum, J.A.M., Mukherjee, U. and Latzko, E. (1987) Plant Physiol. 84, 182–187.
[20] Ap Rees, T., Morrel, S., Edwards, J., Wilson, P.M. and Green, J.H. (1985) in: Regulation of Carbohydrate Paritioning in Photosynthetic Tissues (Heath, R.L. and Priess, J. eds) pp. 129–158, Waverly, Baltimore.
[21] Huber, S.C. and Akazawa, T. (1986) Plant Physiol. 81, 1008–1013.
[22] Sung, S.J.S., Xu, D.-P., Galloway, C.M. and Black, C.C. (1988) Physiol. Plant. 72, 650–654.
[23] Huseman, W. and Barz, W. (1977) Physiol. Plant. 40, 77–81.
[24] Stitt, M. and Heldt, H.W. (1981) Plant Physiol. 68, 755–761.
[25] Stitt, M. and Heldt, H.W. (1985) Planta 164, 179–188.
[26] Lilley, R.M.C., Stitt, M., Gerhard, M. and Heldt, H.W. (1982) Plant Physiol. 70, 965–970.
[27] Dennis, D.T. and Greyson, M. (1987) Physiol. Plant. 69, 395–404.
[28] Kombrink, E. and Kruger, N.J. (1984) Z. Pflanzenphysiol. 114, 443–453.
[29] Stitt, M. (1989) Plant Physiol. 89, 628–633.
[30] Stitt, M., Cseke, C. and Buchanan, B.B. (1985) Physiol. Veg. 23, 819–827.
[31] Stitt, M., Gerhardt, R., Wilke, I. and Heldt, H.W. (1987) Physiol. Plant. 69, 377–386.
[32] Dancer, J. and Ap Rees, T. (1989) Planta, in press.